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Probing Structure-Function Relations in Heme-Containing Oxygenases and Peroxidases

JOHN H. DAWSON

Structural factors that influence functional properties are examined in the case of four heme enzymes: cytochrome P-450, chloroperoxidase, horseradish peroxidase, and secondary amine mono-oxygenase. The identity of the axial ligand, the nature of the heme environment, and the steric accessibility of the heme iron and heme edge combine to play major roles in determining the reactivity of each enzyme. The importance of synthetic porphyrin models in understanding the properties of the protein-free metal center is emphasized. The conclusions described herein have been derived from studies at the interface between biological and inorganic chemistry.

NATURE USES METAL IONS EXTENSIVELY IN FUNCTIONAL AS well as structural roles. As biological catalysts, metal ions are of crucial importance in electron transfer reactions and in the activation and transport of small molecules such as dioxygen (O_2). Two major factors control the properties of metal ions in biological systems: (i) the structure of the metal, including the geometry of the complex and the nature of the ligands attached to the metal and (ii) the environment of the metal complex, including the polarity of the immediate surroundings and steric constraints on the accessibility of substrates to the metal and of the metal to the solvent. It was once thought that most, if not all, metal centers in enzymes had anomalous properties relative to isolated abiological metal complexes and were therefore in "entatic" or tensed states "poised for catalytic action" (*1*). Most metal complexes in proteins have structures that can be accurately reproduced in small molecule analogs outside the protein (*2*). Nonetheless, the protein environment clearly plays a crucial role in controlling the reactivity of the metal, and in some cases the protein can force metal ions into unusual geometries; the protein environment may be the determining factor controlling the activity of the increasing number of functionally distinct metalloproteins that have essentially identical metal centers. For example, cytochrome P-450 and chloroperoxidase have identical thiolate-ligated heme iron active sites, but P-450 is a mono-oxygenase, whereas chloroperoxidase halogenates substrates. Obviously, the final catalytic activity of a metal-containing system depends both on the structure of the metal and on the influence of the protein environment.

The importance of copper, iron, and zinc in biological systems has been evident for more than 50 years (*3*). The frequent requirement for other metals including cobalt, manganese, molybdenum, nickel, and vanadium in biochemical processes has been recognized (*4*). Even selenium has been found to be essential for the function of certain enzymes (*5*). In the late 1960s many inorganic chemists shifted from studying "pure" inorganic chemistry to investigating the biochemistry of metals. No longer were metalloproteins just proteins that happened to contain a metal. Instead, the metal became the focus of attention, the "built-in" probe.

In this article I address the question of how structure influences function in a system where enough is known about structure to begin to get answers to the questions. I examine results for four heme enzymes, cytochrome P-450, chloroperoxidase, horseradish peroxidase, and secondary amine mono-oxygenase, which have a number of common metal coordination structural features but different functional properties. The breadth of the field and lack of space prevent discussion of several "hot" bio-inorganic subjects such as metal-DNA interactions (*6*); the recent discovery of vanadium-containing enzymes (*7*); the involvement of nickel (*8*), manganese (*9*), and molybdenum (*10*) in the mechanisms of action of a growing number of metalloenzymes; and the role of copper (*11*) and nonheme iron in O_2 binding and activation (*12*), to name just a few.

The author is a professor in the Department of Chemistry, University of South Carolina, Columbia, SC 29208.

Heme-Containing Oxygenases and Peroxidases: General Properties

The ability of heme enzymes to incorporate oxygen atoms from O_2 into organic substrates (the oxygenase activity) and to use H_2O_2 and other peroxides to oxidize substrates (the peroxidase activity) amply illustrates the catalytic versatility of heme-containing enzymes. The best characterized mono-oxygenase (*13*) containing a heme prosthetic group is cytochrome P-450 (*14–20*). Horseradish peroxidase is the most thoroughly investigated heme-containing peroxidase (*21, 22*). Chloroperoxidase has spectral properties that are similar to those of P-450, and, as a peroxidase, it has many functional similarities to horseradish peroxidase (*14, 23*). Chloroperoxidase is different from essentially all other peroxidases in that it can chlorinate hydrocarbons in a peroxide-dependent manner. Finally, a second heme-containing mono-oxygenase has recently been purified to homogeneity (*24*). This new enzyme, secondary amine mono-oxygenase, has spectral properties that most closely parallel those of imidazole-ligated heme systems such as horseradish peroxidase (*25*) and yet, like P-450, it is a mono-oxygenase. A complete explanation of how such diverse reactions can originate from a single type of prosthetic group, the heme, requires knowledge of both the proximal axial iron ligand and the nature of the protein environment on both the proximal and distal sides.

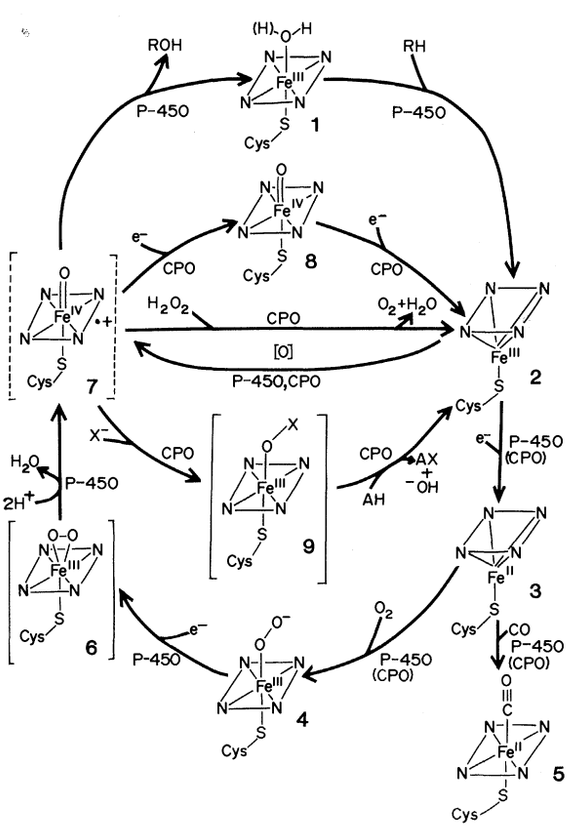

Fig. 1. Catalytic cycles of cytochrome P-450 and chloroperoxidase (CPO) and the postulated structures of the intermediates. The particular reactions that each enzyme catalyzes are indicated. Reactions that chloroperoxidase will catalyze that are not part of its reactions cycles are labeled (CPO). Structure **4** is shown as a complex of Fe^{III} and superoxide anion although it could also be described as an adduct of Fe^{II} and neutral O_2. States **6** and **9** are hypothetical intermediates whose structures have not been established. Structure **7** is a hypothetical intermediate in the P-450 cycle but has been established to be an intermediate in the chloroperoxidase path. The dianionic porphyrin macrocycle is abbreviated as a parallelogram with nitrogens at the corners. Structures **1**, **2**, and **7** are neutral, the overall charge on structures **3**, **4**, **5**, **8**, and **9** is −1, and the charge on structure **6** is −2.

Cytochrome P-450

Enzymes in the cytochrome P-450 family are remarkably versatile O_2-activating catalysts that can incorporate one of the two oxygen atoms of O_2 into a broad variety of substrates with concomitant reduction of the other oxygen atom by two electrons to water (*14–20*). In addition to the conversion of unactivated alkanes to alcohols (Eq. 1),

$$R\text{–}H + O_2 + 2H^+ + 2e^- \rightarrow R\text{–}OH + H_2O \quad (1)$$

P-450 enzymes transfer oxygen atoms to a wide range of substrates, transforming alkenes to epoxides, arenes to phenols, and sulfides to sulfoxides and then to sulfones. P-450 enzymes also oxidatively cleave C–N and C–O bonds in the metabolism of amines and ethers, respectively, and C–C bonds in the biosynthesis of steroid hormones. Under anaerobic conditions, P-450 will reductively dehalogenate halocarbons to the corresponding alkanes.

P-450 enzymes are generally membrane bound and have been found in plants, animals, yeasts, and bacteria (*14–20*). The best characterized P-450 enzyme is the soluble camphor-metabolizing P-450–CAM isolated from *Pseudomonas putida*. All P-450 enzymes share a common reaction cycle with four well-characterized intermediates (**1** → **4**, Fig. 1) beginning with the low-spin six-coordinate Fe^{III} resting state (**1**). Substrate binding yields the high-spin five-coordinate derivative (**2**), which has a higher reduction potential than state **1** to facilitate electron transfer and give the high-spin five-coordinate deoxy-ferrous enzyme (**3**). Addition of O_2 produces the last stable intermediate, oxy–P-450 (**4**). The oxygenated organic product and water are formed after addition of the second electron to oxy–P-450. It is generally thought that at least two species (**6** and **7**) precede oxo-oxygen transfer (*26*), with water being formed upon cleavage of the O–O bond of the bound peroxide (**6** → **7**). The O–O bond cleavage step and the nature of state **7** will be discussed in greater detail below.

An alternative reaction path, called the peroxide shunt, has been discovered (*14–20*). In this pathway, addition of various oxygen atom donors such as iodosobenzene and amine oxides to the high-spin Fe^{III} substrate-bound enzyme leads directly to state **7** and then to oxo transfer. Numerous mechanistic studies have led to the conclusion that, for alkane hydroxylation, the oxo transfer step proceeds with **7** abstracting a hydrogen atom from the substrate to yield a carbon radical and iron-bound hydroxyl radical followed by radical recombination to form the alcohol (*14–19, 27*). There is no direct evidence for the existence of either **6** or **7** as intermediates in the mechanism of action of P-450. In order to emphasize the structural similarities between certain P-450 and chloroperoxidase derivatives, the reaction cycles of the two enzymes are superimposed in Fig. 1.

Extensive and well-documented spectral comparisons of heme protein and synthetic porphyrin complexes of known structure with P-450 states **1** through **4** as well as with Fe^{II}-CO P-450 (**5**) have provided conclusive evidence for cysteinate axial ligation in all five derivatives (*14, 15*). This evidence has come from a large number of laboratories and rests primarily on results obtained with spectroscopic techniques such as magnetic circular dichroism (MCD), extended x-ray absorption fine structure (EXAFS), and resonance Raman (*28*) as well as magnetic resonance methods (*20*). The first direct observation of sulfur donor ligation came from the use of EXAFS spectroscopy. Through the collaborative efforts of the Dawson and Hodgson laboratories (*29–31*), all five stable P-450 states have been studied with this technique. Curve fitting analysis of the data revealed the presence of a sulfur donor ligand to each derivative as listed in Table 1 together with x-ray crystallography data for relevant model complexes (*32–34*).

Recently, the x-ray crystal structures of P-450–CAM states **1** and **2** have been reported by Poulos *et al.* (*35, 36*). The Fe–S (axial) bond lengths reported for these two P-450 states (Table 1) agree quite well with the values determined previously by EXAFS. Of particular importance, the crystal structure shows that the heme prosthetic group is very deeply buried within the enzyme and that the substrate binding pocket is very nonpolar with an absence of amino acid residues that could participate in O_2 activation. As far as the heme iron coordination structure is concerned, the crystal structure of P-450 does not contain any surprises. This statement is not meant to belittle the importance of the crystal structure in improving our understanding of O_2 activation, especially by providing information about the proximal and distal heme environments. Rather, it adds emphasis to the remarkably accurate description of the active-site heme iron of P-450 that has emerged from extensive investigations of the enzyme coupled with equally elaborate studies of synthetic model complexes and indicates the level of success such a concerted effort of bio-inorganic chemists can achieve.

Chloroperoxidase

Chloroperoxidase, like P-450, is a rather versatile catalyst (*14, 23*). As illustrated in Fig. 1, it can function as a peroxidase (**2** → **7** → **8** → **2**) by oxidizing substrates with simultaneous reduction of H_2O_2 to H_2O, as a catalase (**2** → **7** → **2**) by disproportionating H_2O_2 to O_2 and H_2O, and as a halogenation catalyst (**2** → **7** → **9** → **2**; Eq. 2).

$$AH + X^- + H^+ + H_2O_2 \rightarrow AX + 2H_2O \quad (2)$$

Unlike other heme-containing peroxidases that can brominate or iodinate halogen acceptors such as β-diketones using the halide anion as the halogen source, chloroperoxidase chlorinates substrates using either chloride (Eq. 2) or chlorite as the halogen source (*14*), in the latter case without the need for H_2O_2.

Recently, chloroperoxidase has been found to be capable of some P-450–type reactions such as the *N*-dealkylation of alkylamines, the epoxidation of alkenes, and the conversion of sulfides to sulfoxides (*37–40*). In the latter two cases, the oxygen atom transferred is derived from H_2O_2 (hence the term, peroxygenation) (*39, 40*). Thus chloroperoxidase can carry out one-electron (peroxidase) as well as two-electron (peroxygenase) reactions. Ortiz de Montellano and co-workers (*39*) showed that the mechanisms of oxo transfer to styrene by chloroperoxidase and P-450 are similar; both proceed without any detectable loss of stereochemistry with *trans*-[1-^{2}H]styrene as the substrate. They concluded that epoxidation of styrene took place by direct interaction of the substrate with the iron-bound oxo atom. In contrast, Fisher and co-workers (*41*) observed a lack of stereoselectivity in the sulfoxidation of methionine and in the chlorination of a chiral cyclopentanedione. The lack of stereoselectivity in these two reactions could mean that they do not involve direct interaction at the iron-oxo group.

Although Fe^{II} derivatives of chloroperoxidase can be prepared, the reaction cycles of the enzyme involve only Fe^{III} and higher oxidation states (Fig. 1). In each of the three reaction modes outlined above, the first step involves addition of H_2O_2 to the high-spin Fe^{III} resting state (**2**) to produce a two-electron oxidized iron-oxo intermediate known as compound I (**7**). In the peroxidase reaction path, compound I is reduced back to the Fe^{III} resting state in two steps through a second high-valent iron-oxo species, compound II (**8**). The structures of **7** and **8** will be discussed in more detail in the section below on horseradish peroxidase. In the catalase reaction mode, state **7** oxidizes a second molecule of H_2O_2 to O_2, which returns the heme iron to its resting state (**2**). In the halogenation pathway, the halide ion may interact directly with the oxo-oxygen of compound I to produce "compound X" (**9**), a ferric hypochlorite adduct (*42*). However, the exact details of how chloroperoxidase halogenates hydrocarbons have not been well established.

The coordination structure of the heme iron of chloroperoxidase, like that of P-450, has been the subject of extensive research over the past 15 years. As has been recently reviewed (*14*), a consensus has been reached that chloroperoxidase also has a thiolate axial ligand (Fig. 1). This conclusion was reached on the basis of investigations similar to those carried out with P-450, that is, spectral comparisons of heme proteins and models with various chloroperoxidase states. Initial evidence that chloroperoxidase lacked a free cysteine to coordinate to the heme iron has recently been ruled out after the determination by Hager and co-workers of the amino acid sequence directly from the gene (*43*). Two chloroperoxidase derivatives, the high-spin ferric and oxy-ferrous states, were examined by EXAFS spectroscopy and were found to contain a sulfur-donor axial ligand (Table 1). Although the EXAFS technique does not directly determine the type of sulfur donor ligand bound to the heme iron, in all of the P-450 and chloroperoxidase states so far investigated, the Fe–S (axial) bond length has been found to be less than or equal to the analogous Fe–S bond length in thiolate-ligated model complexes that have been crystallographically characterized. This is most consistent with cysteinate rather than cysteine axial ligation (*44*). The difference in Fe–S bond lengths of 0.07 Å for the two high-spin

Table 1. Structural details for cytochrome P-450, chloroperoxidase, and relevant, porphyrin model complexes. All parameters are from EXAFS measurements except where noted and were obtained from curve fitting. Abbreviations: LM2, liver microsomal band 2; TPP, tetraphenylporphyrin; PPIXDME, protoporphyrin IX dimethyl ester; SEt, ethanethiolate; TpPP, mesotetrakis (α,α,α,α-*o*-pivalamidophenyl)porphyrin. *N* indicates the number of atoms at the distance indicated.

System	Fe–N (porphyrin)		Fe–S (axial)		Reference
	R (Å)	*N*	*R* (Å)	*N*	
Low-spin Fe^{III}					
P-450–CAM	2.00	5.0	2.22	0.6	(*30*)
P-450–LM2	2.00	4.8	2.19	0.8	(*29*)
P-450–CAM*†		4	2.20	1	(*35*)
Fe(TPP)(HSC_6H_5)(SC_6H_5)*		4	2.27(RS^-)	1	(*32*)
			2.43(RSH)	1	
High-spin Fe^{III}					
P-450–CAM	2.06	5.2	2.23	0.8	(*30*)
Chloroperoxidase	2.05	4.2	2.30	0.9	(*29*)
P-450–CAM*‡	2.05	4	2.20	4	(*35*)
Fe(PPIXDME)($SC_6H_5NO_2$)*	2.064	4	2.324	1	(*33*)
High-spin Fe^{II}					
P-450–CAM	2.08	3.0	2.34§	0.6	(*30*)
Fe(TPP)(SEt)*	2.096	4	2.360	1	(*34*)
Low-spin Fe^{II}-O_2 complex					
P-450–CAM‖	2.00	7.8¶	2.37	1.3	(*31*)
Chloroperoxidase#	2.00	7.4¶	2.37	1.4	(*31*)
Fe(TpPP)(SC_6HF_4)(O_2)*	1.990	4	2.369	1	(*34*)
Low-spin Fe^{II}-CO complex					
P-450–CAM	1.98	3.3	2.32	1.0	(*30*)
Fe(TPP)(SEt)(CO)*	1.993	4	2.352	1	(*34*)

*Data from crystal structure determination. †Data with a resolution of 2.2 Å. The iron is 0.29 Å out of the plane of the four pyrrole nitrogens toward the cysteinate axial ligand. The sixth ligand is water (or hydroxide). ‡Data with a resolution of 1.7 Å. The iron is 0.43 Å out of the plane of the four pyrrole nitrogens toward the cysteinate axial ligand. There is no sixth ligand. The $C_\beta S_\gamma Fe$ bond angle is 105.9°. §Best fit to filtered EXAFS data. $Fe–S_{axial}$ = 2.38 Å for unfiltered EXAFS data. ‖Fe–O (dioxygen) = 1.78 Å; $N(O_{axial})$ = 1.1. ¶Analysis of low-temperature data using parameters derived from the study of model complexes at room temperature may result in high *N* values due to Debye-Waller effects [see (*31*)]. #Fe–O (dioxygen) = 1.77 Å; $N(O_{axial})$ = 1.3.

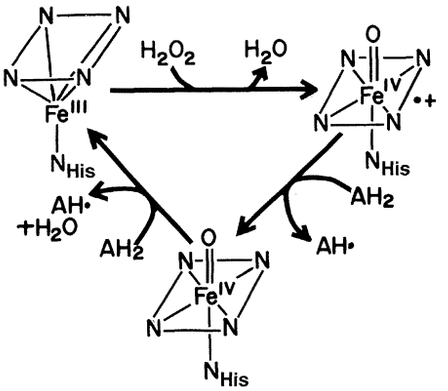

Fig. 2. Catalytic cycle of horseradish peroxidase. The overall charge on the ferric state (top left) and on compound I (top right) is +1, whereas compound II (bottom center) is neutral. See the legend to Fig. 1 for additional information.

Table 2. Structural details for high-valent iron-oxo states of horseradish peroxidase (HRP) and relevant porphyrin model complexes. All parameters are from EXAFS measurements and were obtained from curve fitting except for the last entry the data for which are from a crystal structure determination. The range of data reported for the EXAFS data results from the use of two different analysis methods.

System	Fe–N (porphyrin) (Å)	Fe–O (oxo) (Å)	Reference
HRP compound I	1.99 –2.00	1.61 –1.64	(*22*)
Groves HRP-I model	2.02 –2.04	1.62 –1.66	(*22*)
HRP compound II	1.99 –2.00	1.60 –1.64	(*22*)
Balch HRP-II model	2.02 –2.03	1.64 –1.66	(*22*)
Weiss HRP-II model	2.005	1.604	(*73*)

Fe^{III} enzyme states (Table 1) is greater than the expected error limit of ±0.02 Å for the EXAFS measurements and may be in accord with the subtle differences observed in physical properties of the two high-spin Fe^{III} species. The Fe–N (porphyrin) bond distances (Table 1) together with knowledge of the spin state provide evidence for the coordination numbers of the various enzyme derivatives examined (*45*); bond distances near 2.00 Å in low-spin complexes are indicative of six-coordination (states **1**, **4**, and **5**), whereas bond distances greater than 2.05 Å in high-spin cases are attributable to five-coordination (states **2** and **3**). Using resonance Raman spectroscopy, Champion and co-workers have directly observed an Fe–S vibration in high-spin Fe^{III} chloroperoxidase (*46*), like that in P-450 (*28*).

Given the rather different catalytic activities of P-450 and chloroperoxidase, the close similarities in active-site structures seems a bit surprising. Additional factors other than just the metal coordination structure play important roles in determining the enzymatic activities. Recent studies by Sono *et al.* have revealed differences in the heme environment of chloroperoxidase relative to P-450 that may correlate with their catalytic differences (*47*). Examination of the ligand binding properties of Fe^{III} and Fe^{II} chloroperoxidase over a wide *p*H range demonstrated that a charged amino acid is present, which influences ligand binding and could also play a role in catalysis. Similar *p*H effects have been seen with catalase and with horseradish and cytochrome c peroxidases (*48–50*). With Fe^{II} chloroperoxidase, a pK_a of 5.5 (where K_a is the acid constant) was found for an amino acid in the vicinity of the active site that influenced the binding of neutral ligands; Hager and co-workers had earlier published similar conclusions about CO binding (*51*). The crystal structure of cytochrome c peroxidase includes a distal histidine that is thought to participate in cleavage of the peroxide O–O bond (*52*). This suggests that the amino acid with a pK_a of 5.5 in chloroperoxidase could also be a histidine. An additional observation in favor of a more polar environment for the heme iron in chloroperoxidase comes from the styrene epoxidation study of Ortiz de Montellano and co-workers (*39*). They found substantially more phenylacetaldehyde formation during epoxidation of styrene by chloroperoxidase than with P-450 and interpreted this as resulting from a 1,2-rearrangement process that would be favored by a more polar environment. Thus chloroperoxidase may have a more polar peroxidase-type heme environment to go with its P-450–type metal coordination structure (*47*).

Horseradish Peroxidase

Horseradish peroxidase catalyzes the oxidation of organic substrates with H_2O_2 as the ultimate electron acceptor (Eq. 3).

$$AH_2 + H_2O_2 \rightarrow A + 2H_2O \quad (3)$$

As with chloroperoxidase, the first step of the peroxidase reaction path involves the addition of H_2O_2 to the Fe^{III} resting state to form a high-valent iron-oxo derivative known as compound I, which is formally two oxidation equivalents above the Fe^{III} state (Fig. 2). Compound I is then reduced back to the Fe^{III} state in two steps through compound II.

Horseradish peroxidase, like chloroperoxidase, catalyzes some P-450–type reactions including the *N*-dealkylation of alkylamines, the hydroxylation of a benzylic methyl group, and the conversion of sulfides to sulfoxides (*37, 39, 40*). However, in all cases except one (*40*), the source of the incorporated oxygen atom is not H_2O_2 (*37, 39*); thus the reaction is not thought to proceed through oxo transfer from compound I. Additional mechanistic distinctions between the *N*-dealkylation of amines by horseradish peroxidase and that catalyzed by P-450 and chloroperoxidase have been probed by Miwa, Hollenberg, and co-workers (*53*), who determined intramolecular isotope effects for the reaction using *N*-methyl,*N*-trideuteriomethylaniline as the substrate. With P-450 and chloroperoxidase, the value is fairly low (1.7 to 3.1), whereas horseradish peroxidase and a few other imidazole-ligated heme proteins gave rise to much larger values (8.6 to 10.1). Horseradish peroxidase also catalyzes some oxygen-incorporating reactions, including the epoxidation of styrene, which require a cosubstrate (co-oxidations) and involve the one-electron oxidation of the cosubstrate to a radical that reacts with O_2 to form a peroxy radical (ROO·), which is responsible for product formation (*54*).

The two high-valent horseradish peroxidase intermediates have been extensively studied (*21, 22*), and the structures shown in Fig. 2 represent the general consensus. Mössbauer spectra of the two derivatives are similar to each other and have been interpreted in terms of a Fe^{IV} oxidation state for both (*55, 56*). Magnetic susceptibility data on compound I (*57*) indicate the presence of three unpaired electrons, as would result from the ferromagnetic coupling of low-spin Fe^{IV} ($S = 1$) with a porphyrin π-cation radical ($S = 1/2$). The π-cation radical assignment is also consistent with ultraviolet-visible absorption and magnetic resonance measurements (*58–60*). Labeling studies have established that a single oxygen atom is bound to the heme iron of each intermediate (*61, 62*). For compound I the oxygen has generally been proposed to be an oxo-oxygen, and both oxo and hydroxyl assignments have been made for compound II. Resonance Raman studies of compound II by Terner *et al.* (*63*) and Kitagawa and co-workers (*64*) argue strongly in favor of the oxo formulation.

To address this problem more quantitatively, we have used EXAFS spectroscopy (*22, 65*) to examine both of the high-valent horseradish peroxidase intermediates as well as synthetic analogs for each state prepared by Groves, Balch, and co-workers (*66, 67*). In all four cases (Table 2), very short Fe–O bond distances of ~1.64 Å have been found that are consistent with the Fe^{IV}=O structures

shown in Fig. 2. Chance *et al.* have also investigated the two high-valent peroxidase states with EXAFS and are in agreement that compound I has a short Fe–O bond length (*68*). However, their spectral data for compound II are clearly different from our data; they conclude that the Fe–O bond length in compound II is about 1.9 Å. More recently, evidence for short Fe–O bond lengths in compound II–type species generated by the addition of peroxides to myoglobin and cytochrome c peroxidase have been reported from resonance Raman and EXAFS spectroscopy and, in the latter system, by x-ray crystallography as well (*69–72*). Preliminary x-ray structural analysis of a synthetic model for compound II by Weiss and co-workers gave a Fe–O bond length of 1.604 Å (*73*). Thus, except for the EXAFS result of Chance *et al.* (*68*) on horseradish peroxidase compound II, the evidence supports the assignment that both compounds I and II have iron-oxo [Fe^{IV}=O] structures.

The inability, with one possible exception (*40*), of horseradish peroxidase compound I to catalyze oxo transfer reactions to suitable substrates is somewhat puzzling, especially in view of the facility with which synthetic compound I models catalyze the reaction with no apparent dependence on the ligand trans to the oxo atom (*17*). Once again, additional factors other than just the metal coordination structure must play a role in controlling the modes of reaction of the enzyme intermediates. Elegant recent studies by Ortiz de Montellano and co-workers of the mechanism-based inactivation of the enzyme argue strongly that the site of reaction of most, if not all, horseradish peroxidase reactions is the heme edge (*39, 74, 75*). Reaction of the peroxidase with aryl- or alkylhydrazines led to covalent attachment at the meso carbon at the heme edge of the porphyrin prosthetic group, whereas heme proteins with accessible metal centers are inactivated by formation of a σ-bonded intermediate which rearranges to form an *N*-alkylated heme derivative that is not seen with horseradish peroxidase. Thus steric factors may actually prevent substrates from getting near enough to the iron-oxo group of horseradish peroxidase compound I to permit oxo transfer to occur (*39, 74, 75*).

Secondary Amine Mono-Oxygenase

Secondary amine mono-oxygenase, first identified and partially purified in the late 1960s by Eady and Large (*76*), and cytochrome P-450 are the only known heme-containing mono-oxygenases. Recently, Alberta and Dawson reported the purification to homogeneity and initial physical characterization of secondary amine mono-oxygenase (*24*), which catalyzes the *N*-dealkylation of dialkylamines, with dimethylamine being the preferred substrate. Methylamine and formaldehyde are products (Eq. 4):

$$(CH_3)_2NH + O_2 + NAD(P)H + H^+ \rightarrow CH_3NH_2 + CH_2O + H_2O + NAD(P^+) \quad (4)$$

where NAD(P)H is the reduced form of $NADP^+$, nicotinamide adenine dinucleotide (phosphate). *N*-dealkylation reactions that are catalyzed by secondary amine mono-oxygenase and P-450 depend directly on O_2 and are inhibited by CO, as would be expected for heme-containing mono-oxygenases. The same reaction is catalyzed by various peroxidases (*37*) but is initiated by H_2O_2 and, since Fe^{II} heme intermediates are not formed, is not inhibited by CO.

In contrast to P-450, secondary amine mono-oxygenase is an oligomeric protein and contains bound flavin and nonheme iron cofactors in addition to the heme prosthetic group (*24*). It is not known whether the enzyme will catalyze other oxygen transfer reactions from among the long list of such reactions carried out by P-450. A reaction cycle for the enzyme has not yet been worked out, although it has been possible to generate ferric, deoxyferrous, and oxy-ferrous derivatives, so that a pathway for O_2 activation such as has been established for P-450 (Fig. 1) may be operative.

Table 3. Soret absorption maxima (wavelength λ in nanometers) and extinction coefficients ε (mM^{-1} cm^{-1}, per heme) for secondary amine mono-oxygenase (SAMO), myoglobin, and cytochrome P-450 (*25*).

Species	SAMO*		Myoglobin†		P-450–CAM‡	
	λ	ϵ_{mM}	λ	ϵ_{mM}	λ	ϵ_{mM}
Ferric	408	(185)	409.5	(157)	391	(102)
Ferric-CN	421	(137)	423	(110)	439	(79)
Ferric-N_3	420	(146)	420	(123)	427	(81)
Ferrous-CO	423	(200)	423	(187)	446	(120)
Ferrous-O_2	418	(140)	418	(128)	419	(82)

*pH 6.8. †pH 7.0. ‡pH 7.4.

Table 4. EPR g values (at 10 K) for secondary amine mono-oxygenase (SAMO), myoglobin, and cytochrome P-450 (*25*).

Enzyme	Low-spin, azide-bound			High-spin, native	
	g_z	g_y	g_x	g_x/g_y	g_z
SAMO*	2.76	2.23	1.79	6.00	2.04
Myoglobin†	2.82	2.19	1.78	6.10	2.01
P-450–CAM‡	2.53	2.25	1.88	8.0, 4.0	1.81

*pH 6.8. †pH 7.0. ‡pH 7.4.

Several derivatives of purified secondary amine mono-oxygenase have been investigated by ultraviolet-visible absorption and electron paramagnetic resonance (EPR) spectroscopy (*25*). The key spectral data for the enzyme together with results for analogous myoglobin and P-450 states are listed in Tables 3 and 4. The spectral properties of the various secondary amine mono-oxygenase species are consistently similar to those of the parallel states of myoglobin and distinct from those of P-450. Like myoglobin and unlike P-450, secondary amine mono-oxygenase forms a stable oxy-ferrous derivative. The spectroscopic similarities between secondary amine mono-oxygenase and myoglobin strongly suggest that the new mono-oxygenase has a histidine-ligated heme iron center as is present in myoglobin.

Heme-Containing Oxygenases and Peroxidases: Structure-Function Relations

Of the four heme enzymes that I have considered, two are oxygenases and two are peroxidases. Interestingly, one of the oxygenases and one of the peroxidases has a thiolate-ligated metal center and in each case the other enzyme of the pair is histidine ligated. All four of the enzymes will *N*-dealkylate alkylamines, and, except for secondary amine mono-oxygenase which has not been tested yet, all will convert sulfides to sulfoxides. Of course, the mono-oxygenases carry out these reactions through an O_2-dependent, CO-inhibited path, whereas in the case of the peroxidases the reactions are initiated by H_2O_2 and are not inhibited by CO. Both peroxidases are known to form compound I as an essential intermediate in their reaction cycles. P-450 is generally assumed to go through a compound I intermediate, and secondary amine mono-oxygenase may as well.

In addition to these similarities among the four enzymes, there are many more differences. Only P-450 is capable of hydroxylating unactivated alkanes, and only chloroperoxidase chlorinates hydrocarbons using Cl^- as the source of the halogen. Although horseradish peroxidase can carry out some P-450–type reactions, upon closer mechanistic scrutiny it is not as clear that these reactions involve

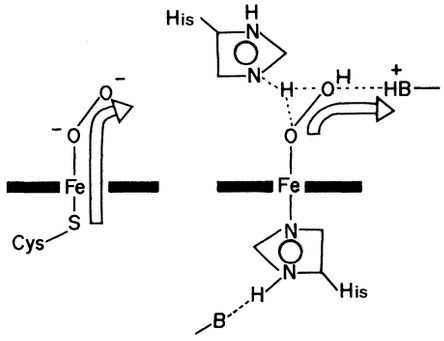

Fig. 3. A schematic view of the "push-pull" mechanism for O–O bond cleavage of an iron-bound peroxide in thiolate-ligated (left) and histidine-ligated [right, adapted from (*36*)] systems such as cytochrome P-450 and horseradish peroxidase, respectively.

direct oxo transfer from a high-valent iron oxo intermediate as likely occurs with P-450. Too little is known about secondary amine mono-oxygenase to generalize about its reactivity.

In trying to understand the similarities and differences among these four enzymes, two factors play dominant roles: the identity of the protein-derived axial ligand and the heme environment. With P-450 and the peroxidases, generation of the high-valent iron-oxo intermediate involves cleavage of the O–O bond of the iron-bound peroxide moiety. For P-450, it has been proposed (*77*) that the thiolate ligand is crucial to that process by serving as a strong internal electron donor to facilitate cleavage of the bond. Evidence in support of this role for the thiolate has come from the enhanced basicity of ligands bound trans to the endogenous cysteinate of P-450 relative to parallel myoglobin adducts (*14, 15, 78*). This idea is further strengthened by the x-ray structural data on P-450 (*35, 36*). Both the proximal and distal sides of the heme are nonpolar and lack groups capable of hydrogen bonding either to the proximal base (the thiolate) or to the bound peroxide as are found in cytochrome c peroxidase (and, by sequence homology, in horseradish peroxidase) (*36*). In the context of the "push-pull" concept of peroxide activation (*36*), it appears that the "push" (*77, 78*) of the axial thiolate in P-450 is sufficient to cleave the O–O bond without need for a "pull" from charged residues on the distal side (Fig. 3).

As has been extensively discussed by Poulos (*36*), in the case of cytochrome c peroxidase (and, by sequence homology, horseradish peroxidase), the proximal histidine hydrogen bonds more strongly to neighboring groups than in the globins leading to a shorter Fe–N (porphyrin) bond distance. This makes the axial histidine a better electron donor (a better "push") to the heme than in the globins and may also help to stabilize higher oxidation states of the iron during catalysis. At the same time, the distal histidine serves as a proton donor and works together with a charged residue (arginine in the case of cytochrome c peroxidase) to make the distal side substantially more polar than in the globins and to "pull" apart the O–O bond of the bound peroxide by stabilizing the separating charge (Fig. 3). The "push-pull" concept provides two ways to generate the key high-valent iron-oxo intermediate: the thiolate "push" or the arginine "pull." Catalase, which has a proximal tyrosine phenolate ligand (*79*), also fits in with this concept. With the neutral residue asparagine in place of the charged arginine as the polar group working in conjunction with the distal histidine to promote O–O bond cleavage, catalase makes up for the lack of a strong "pull" by having a proximal ligand with a full negative charge and therefore a strong "push."

Once the high-valent iron-oxo state is generated, the heme environment plays a crucial role in controlling the subsequent reactivity of the system. Compound I is both a good oxidant and a good oxo donor. Ortiz de Montellano has shown that with horseradish peroxidase reactions take place at the heme edge and steric factors prevent substrates from getting close enough to the iron-bound oxo group for oxo transfer to occur (*39, 74, 75*). Conversely, with P-450, the heme is buried and the iron-oxo is accessible to the substrate; oxo transfer is the dominant reaction. Chloroperoxidase occupies a middle ground with a P-450 metal coordination structure in a peroxidase environment (*47*). It carries out both one- and two-electron chemistry, possibly at the heme edge and at the iron-oxo group, respectively. The stereospecificity of the oxo transfer chemistry of chloroperoxidase (epoxidation) has implicated direct access to the iron-oxo group (*39*), whereas the lack of stereospecificity in other reactions may be the result of chemistry at the heme edge. In particular, if the chlorination reaction involves chemistry at the heme edge, it would explain why P-450 does not chlorinate hydrocarbons since the heme edge of P-450 is buried. Geigert *et al.* (*80*) have presented evidence against halogenation through an enzyme-bound intermediate such as **9**. Clearly, a consensus has yet to emerge concerning the mechanism of chlorination by chloroperoxidase. It is also possible that multiple mechanism operate.

Finally, we come back to secondary amine mono-oxygenase, the least extensively examined of the four enzymes investigated in this article. With our understanding of the metal coordination structures of the other three enzymes coupled with the influence that the heme environment plays in their reactivities, we can make some predictions about secondary amine mono-oxygenase. It probably has a peroxidase proximal and distal heme environment to promote O–O bond cleavage in the absence of a thiolate ligand. It also is likely to have a buried heme edge to suppress peroxidase reactions and an accessible iron-oxo unit to promote oxo transfer. These predictions are subject to revision as more mechanistic information about the enzyme becomes available.

Conclusions

A wide range of catalytic activities can be achieved in heme enzymes that use O_2 and H_2O_2 through variations in the nature of the proximal axial ligand, in the polarity of the proximal and distal heme environments, and in the degree of accessibility of the heme iron center and the heme edge. Four examples have been described in some detail. Cytochrome P-450 has a thiolate axial ligand, a very nonpolar environment for the heme and for the organic and O_2 substrates, and a buried heme edge to suppress peroxidase-type electron transfer reactions, but it has an accessible heme iron as is necessary for its function as an oxo transfer catalyst. Horseradish peroxidase has an imidazole axial ligand, a charged and polar heme environment to facilitate cleavage of the peroxide O–O bond, a relatively inaccessible heme iron to avoid oxo transfer reactions, and an exposed heme edge to promote electron transfer reactions. The mechanistic activity of chloroperoxidase falls between that of P-450 and that of horseradish peroxidase. Like P-450, this enzyme has a thiolate axial ligand, but, like horseradish peroxidase, it has a more polar heme environment. It can perform some oxo transfer reactions and also some peroxidase electron transfer reactions and thus may be accessible at both the heme iron and the heme edge. Secondary amine mono-oxygenase has only recently been purified to homogeneity, and so much less is known about its structural and functional properties. However, it can carry out some P-450–type chemistry and yet has an imidazole axial ligand. Future experiments with this enzyme will further test our understanding of the relation between structure and function in this class of heme enzymes.

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